NATIONAL BUREAU OF STANDARDS REPORT

10 054

Technical Summary Report Number 2
for the Period
May 1, 1968 to April 30, 1969

Contract NAONR 14-67
ONR Contract Authority Identification Number NR 137-793

SUBJECT

MICROBIAL CORROSION

SUBMITTED TO THE OFFICE OF NAVAL RESEARCH

U.S. DEPARTMENT OF COMMERCE
NATIONAL BUREAU OF STANDARDS

NATIONAL BUREAU OF STANDARDS

The National Bureau of Standards [1] was established by an act of Congress March 3, 1901. Today, in addition to serving as the Nation's central measurement laboratory, the Bureau is a principal focal point in the Federal Government for assuring maximum application of the physical and engineering sciences to the advancement of technology in industry and commerce. To this end the Bureau conducts research and provides central national services in four broad program areas. These are: (1) basic measurements and standards, (2) materials measurements and standards, (3) technological measurements and standards, and (4) transfer of technology.

The Bureau comprises the Institute for Basic Standards, the Institute for Materials Research, the Institute for Applied Technology, the Center for Radiation Research, the Center for Computer Sciences and Technology, and the Office for Information Programs.

THE INSTITUTE FOR BASIC STANDARDS provides the central basis within the United States of a complete and consistent system of physical measurement; coordinates that system with measurement systems of other nations, and furnishes essential services leading to accurate and uniform physical measurements throughout the Nation's scientific community, industry, and commerce. The Institute consists of an Office of Measurement Services and the following technical divisions:

Applied Mathematics—Electricity—Metrology—Mechanics—Heat—Atomic and Molecular Physics—Radio Physics [2]—Radio Engineering [2]—Time and Frequency [2]—Astrophysics [3]—Cryogenics. [3]

THE INSTITUTE FOR MATERIALS RESEARCH conducts materials research leading to improved methods of measurement standards, and data on the properties of well-characterized materials needed by industry, commerce, educational institutions, and Government; develops, produces, and distributes standard reference materials; relates the physical and chemical properties of materials to their behavior and their interaction with their environments; and provides advisory and research services to other Government agencies. The Institute consists of an Office of Standard Reference Materials and the following divisions:

Analytical Chemistry—Polymers—Metallurgy—Inorganic Materials—Physical Chemistry.

THE INSTITUTE FOR APPLIED TECHNOLOGY provides technical services to promote the use of available technology and to facilitate technological innovation in industry and Government; cooperates with public and private organizations in the development of technological standards, and test methodologies; and provides advisory and research services for Federal, state, and local government agencies. The Institute consists of the following technical divisions and offices:

Engineering Standards—Weights and Measures — Invention and Innovation — Vehicle Systems Research—Product Evaluation—Building Research—Instrument Shops—Measurement Engineering—Electronic Technology—Technical Analysis.

THE CENTER FOR RADIATION RESEARCH engages in research, measurement, and application of radiation to the solution of Bureau mission problems and the problems of other agencies and institutions. The Center consists of the following divisions:

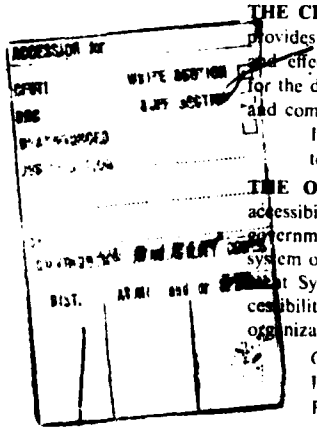
Reactor Radiation—Linac Radiation—Nuclear Radiation—Applied Radiation.

THE CENTER FOR COMPUTER SCIENCES AND TECHNOLOGY conducts research and provides technical services designed to aid Government agencies in the selection, acquisition, and effective use of automatic data processing equipment; and serves as the principal focus for the development of Federal standards for automatic data processing equipment, techniques, and computer languages. The Center consists of the following offices and divisions:

Information Processing Standards—Computer Information — Computer Services — Systems Development—Information Processing Technology.

THE OFFICE FOR INFORMATION PROGRAMS promotes optimum dissemination and accessibility of scientific information generated within NBS and other agencies of the Federal Government; promotes the development of the National Standard Reference Data System and a system of information analysis centers dealing with the broader aspects of the National Measurement System, and provides appropriate services to ensure that the NBS staff has optimum accessibility to the scientific information of the world. The Office consists of the following organizational units:

Office of Standard Reference Data—Clearinghouse for Federal Scientific and Technical Information —Office of Technical Information and Publications—Library—Office of Public Information—Office of International Relations.



[1] Headquarters and Laboratories at Gaithersburg, Maryland, unless otherwise noted; mailing address Washington, D.C. 20234.
[2] Located at Boulder, Colorado 80302.
[3] Located at 5285 Port Royal Road, Springfield, Virginia 22151.

NATIONAL BUREAU OF STANDARDS REPORT

NBS PROJECT
3120442

NBS REPORT
10 054

Technical Summary Report Number 2
for the Period
May 1, 1968 to April 30, 1969

Contract NAONR 14-67
ONR Contract Authority Identification Number NR 137-793

Subject

MICROBIAL CORROSION

Submitted to the Office of Naval Research
by

Warren P. Iverson and Joan Calvert
Corrosion Section
National Bureau of Standards
Washington, D. C. 20234

U.S. DEPARTMENT OF COMMERCE
NATIONAL BUREAU OF STANDARDS

ABSTRACT

An agar medium which will support good surface growth of marine sulfate reducers (Desulfovibrio) has been devised. Using this medium, a marine isolate of Desulfovibrio has been obtained in pure culture and used for anaerobic corrosion studies. Using polarization techniques for corrosion rate determinations, it was found that the rate of corrosion of mild steel (1020), in a culture of the same medium minus added ferrous ions, decreased to about 0.1 mdd and then increased to 7-8 mdd before termination. These changes were correlated with the formation and removal of an iron sulfide film.

A non-marine strain of Desulfovibrio was found to produce a volatile organo-phosphorous compound which reacts with mild steel to form iron phosphide (Fe_2P). It also has the ability to reduce the redox dye, benzyl viologen. The marine isolate also appears to produce this compound.

Growth of the marine strain in a sea water medium containing ferrous ions to which trypticase and phytone were added resulted in formation of a black material, which upon heating to 1,204°C in a vacuum oven, was found to consist of a mixture of FeS and Fe_3P (schreibersite).

TABLE OF CONTENTS

INTRODUCTION

The studies on microbial corrosion described in this report are a continuation of those initiated and reported on in the last Technical Summary Report (1). Emphasis continued to be placed on the mechanism of anaerobic corrosion caused by sulfate-reducing bacteria, primarily those of the genus Desulfovibrio.

One of the objectives of this contract was to obtain a pure culture of a marine species of Desulfovibrio. Successful completion of this objective in addition to the development of an agar medium to support good surface growth has been accomplished.

As a number of mechanisms of anaerobic corrosion by these organisms have been postulated, initiation of corrosion studies by the marine isolate were undertaken to ascertain which one if any, might be of special significance.

In previous studies on the direct cathodic depolarization mechanism of bacterial corrosion, it was found that iron phosphide (Fe_2P) was formed by a non-marine strain of Desulfovibrio as a corrosion product. In the last report, evidence of the formation of a volatile phosphorous containing gas by a Desulfovibrio strain was found. Further studies on this compound are described in this report.

EXPERIMENTAL WORK

1. Growth and Isolation Studies

It has been customary to grow sulfate reducers (Desulfovibrio sp.) in liquid or semi-solid media. This procedure makes it difficult to detect contamination. However, cultivating organisms on the surface of agar enables this problem to be minimized. In the previous report (1) the successful cultivation of non-marine forms on an agar surface was discussed.

(a.) Isolation and Growth of Desulfovibrio from Marine Fouling

Since one of the objectives of this investigation was to study the corrosion mechanism of marine sulfate reducers, it was necessary to obtain and cultivate a pure culture of the organism, preferably on an agar surface.

Previously (1) it was mentioned that difficulty was experienced in isolating marine forms of Desulfovibrio using agar plates of trypticase soy agar (BBL*) plus salts medium (Appendix) to which additional agar was added (final conc. 2%). Although growth of the organisms occurred on this medium, it was always mixed with the growth of contaminating organisms and isolated colonies could not be selectively separated.

This medium had previously been found to be very satisfactory for isolation and growth of non-marine forms of this organism (2).

Impure cultures of a marine Desulfovibrio sp. had been obtained by inoculation of API medium (Appendix) with fouling detritus scraped from pilings off the Atlantic Coast near Virginia Beach, Va.

In preparing the trypticase soy agar plus salts medium, distilled water was used as indicated in the formulation. Increasing the total sodium chloride concentration of the medium to 3% gave no improvement in selective isolation. The use of artificial sea water prepared from an ASTM salt mixture (Appendix), instead of distilled water likewise showed no improvement.

*Baltimore Biological Laboratories

In streaking out, freshly isolated impure cultures in API broth, on the agar medium, some improvement was noted, however, by substituting aged sea water (ca. 1 year old, stored unfiltered in a polyethylene bottle) in place of distilled water. Growth of the contaminants was reduced by elimination of the sodium lactate from the medium. The elimination of magnesium sulfate appeared to have no noticeable effect upon the growth of the sulfate reducers as evidenced by the degree of blackening in the medium surrounding their growth. There appeared to be two types of blackening in the medium. An intense blackening which appeared around the colonies and a lighter blackening throughout the agar as well as on uninoculated plates mixed with the inoculated plates in a Brewer jar or desiccator. The blackening in both cases disappears upon exposure to air. It is suspected that one type may be due to H_2S formation by the organism and the other type to formation of the organo-phosphorous compound, vide infra.

The formulation of the resulting agar medium is presented in the Appendix. A marine strain of Desulfovibrio, as yet unidentified as to species, isolated on this medium was used for the corrosion study in this report. Growth of the isolate on this medium, in a hydrogen atmosphere at 27°C±1.0, follows the streak path of the inoculating loop on the agar surface and is quite extensive.

The purity and identification of the culture as a species of Desulfovibrio was verified by microscopic and visual observation of the growth characteristics. Positive identification was made by flooding the agar surface on which growth was present with an alkaline solution (10% NaOH) and observing a bright red fluorescence of the colonies under ultra violet light ("dark light"). This red fluorescence has been reported by Postgate (2) to be characteristic of cells of organisms in the genus Desulfovibrio.

Colonies of Desulfovibrio on the agar surface may be recognized by their dark centers after the plate has been exposed to the atmosphere. When cells from a colony are streaked over the surface of a plate, blackening of the plate is indicative of growth of the organisms.

Use of this agar medium in conjunction with API medium should greatly facilitate isolation of marine strains of Desulfovibrio. Sea water or other material suspected of containing the organisms may be injected by means of a hypodermic syringe into commercially available vials of API medium at the site of collection. Blackening of this medium in the vial is usually indicative of the presence of Desulfovibric species and it may even occur before the vials are brought into the laboratory depending upon the time, the concentration of the organisms and the temperature. In the laboratory, the culture from vials showing darkening may be removed by a hypodermic syringe and a few drops placed on the surface of the sea water trypticase soy agar. Using the agar streak dilution technique, the organisms on the plate may be separated sufficiently to yield isolate colonies upon incubation of the plates under hydrogen. Positive identification can be made by observation of the red fluorescence of suspected colonies under UV light upon the addition of an alkaline solution to the agar surface.

(b.) Growth of Non-Marine Strains of Desulfovibrio on Trypticase Soy Sea Water plus Agar

Over the past several years, 4 cultures of non-marine strains of Desulfovibrio have been grown in pure culture on plates of trypticase soy broth (BBL) plus agar (Appendix). These four cultures were: a) the API strain (Mid-Continent strain A. Recommended by the American Petroleum Institute for biocide testing), b) a strain isolated from a tubercle in a water main at

Ft. Detrick, Frederick, Maryland, c) a strain isolated from a corrosion pit in an aluminum tank, d) a strain obtained from badly contaminated water in a fuel tank.

Growth on this media is very transparent in appearance and follows the path of the inoculating loop. It is not very luxurient, however. Ordinarily the medium is prepared by adding distilled water to the dehydrated form of the medium, which is then sterilized by autoclaving. If aged sea water was substituted for the distilled water, growth of the four cultures on the agar surface was quite luxurient and tended to be quite opaque and yellow.

On the basis of these limited observations one might be tempted to suggest a marine origin for the so called non-marine forms of these organisms.

2. Physiological Studies

As reported previously (1), it appears that the API strain of Desulfovibrio can affect the corrosion process by removal of hydrogen or electrons on the surface of iron or steel (cathodic depolarization) using phosphate as an electron acceptor. The end corrosion product appears to be a colloidal form of iron phosphide (Fe_2P). In the previous report (1) initial studies on the mechanism of iron phosphide formation were described. A volatile phosphorous compound produced by a non-marine strain of Desulfovibrio (API strain), which did not appear to be phosphine, was identified as being possibly involved in the formation of iron phosphide. Further studies were made on this compound. The marine isolate was found to form a different form of iron phosphide: Fe_3P.

(a.) Identification of Black Product Produced by the Marine Isolate (Desulfovibrio) Growing in the Presence of Ferrous Ions

Since iron phosphide production had been reported for the API strain (1, 4) of Desulfovibrio it was of interest to determine whether it could also be produced by the marine isolate of Desulfovibrio.

Two centrifuge bottles, each containing 185 ml of the trypticase sea water medium (Appendix) were inoculated with a loopfull of the marine strain. This medium is essentially the same as the agar medium used for maintenance of the organisms except that it contained a higher concentration of ferrous ammonium sulfate and of course no agar. Rubber caps with a fine hollow needle through each of them to facilitate gas entry and removal, were placed over the bottle openings and the bottles placed in a vacuum desiccator, which was evacuated and replaced with hydrogen 3 times.

After 6 days incubation at 27°C, the blackened culture fluid in the bottles was rapidly frozen in dry ice to facilitate coalescence of the black colloidal particles. After thawing and centrifugation, the black material was washed twice in an atmosphere of hydrogen and placed under vacuum in a desiccator to remove the moisture. About 880 mg of dry powder was obtained. The material was non magnetic. Upon heating the material to 1,204°C in a vacuum oven for 15 minutes and allowing a day for cooling to ambient temperature, a black powder showing magnetic properties was obtained. X-ray diffraction of this powder indicated the presence of some phosphide. It was not a pure preparation, however, as indicated by the presence of other lines due to FeS in the pattern. Addition of dilute HCl solution to the material produced gas evolution and an odor of H_2S. After H_2S evolution had stopped the resulting black material was washed thoroughly in distilled water and dried

for about ten minutes at 110°C. X-ray diffraction analysis of the material (still magnetic) indicated a structure identical to schreibersite(Fe_3P) of meteoric origin (Cerros de Buen Huerto).

The black precipitate formed by the reaction of the bacteria-free filtrate of the API culture (in 2% yeast extract broth containing steel) on mild steel was found, to be primarily Fe_2P (1), however.

(b.) Oxidation of Black Product Produced by Marine Strain (colloidal iron phosphide)

The black product obtained from the marine isolate, before heat treatment, slowly oxidizes in the presence of moisture and air to a yellow orange color. This has been noticed with previous preparations. X-ray diffraction of this oxidized material indicates that it is most likely in the colloidal state as no distinct pattern for Fe_3P was observed. It is suspected that part of the material may be colloidal ferric phosphate. Further analysis will be made (X-ray diffraction of the vacuum heated material, chemical analysis, etc.) of this and other similar preparations of Fe_3P. After heating, it appears to be resistant to the same type of oxidation (as indicated by an absence of color changes).

(c.) Production of H_2S by Marine Strain

During examination of the blackened trypticase sea water agar plates on which the marine strain had grown, browning of lead acetate paper, held in the vicinity of the plates after dilute acid (sulfuric) had been added to them, was noted.

To determine the nature of this lead compound 8 inoculated plates of trypticase sea water agar (containing 0.05% ferrous ammonium sulfate, added after sterilization) were placed in a Brewer jar with 2 petri plates each containing

10 ml of lead acetate solution. After incubation under a hydrogen atmosphere for 7 days, 124 mg of a silvery appearing material in the form of small flakes was obtained. Qualitative spectrochemical analysis of the material indicated lead as the major element (greater than 10%). No phosphorous was found. A separate chemical analysis indicated the presence of 13.3% sulfur. On the basis of these analyses the material was lead sulfide. This was confirmed by X-ray diffraction. The gas which darkened the lead acetate paper was hydrogen sulfide.

In addition to the formation of hydrogen sulfide in this medium, the organophosphorous compound probably also was produced as evidenced by the formation of Fe_3P, the reduction of benzyl viologen by a culture filtrate of the organism and a mass spectrum quite similar to that of the compound, vide infra.

(d.) Identification of Volatile Phosphorous Compound

Previously (1) it has been determined that the API strain growing on plates of 2% yeast extract agar 2% containing dipotassium phosphate (0.5%) produced a phosphorous containing compound. Attempts to collect any phosphorous containing compound in a liquid nitrogen trap were unsuccessful. In these attempts, hydrogen was passed over inoculated plates of the above medium and through a series of cold traps [2 ice-sodium chloride traps to remove most of the moisture, a methanol-dry ice trap (-80°C) and finally the liquid nitrogen trap (-196°C)].

On examination of the methanol-dry ice trap, however, in subsequent analyses, a compound was collected having a distinct infra-red spectrum as indicated in Figure 1. The absorption peaks are at 694, 815, 920, 1035, 1094 and 1268 cm^{-1}.

These peaks appear to be suggestive of a compound with a P-O type of bond. A peak (not indicated in Figure 1) was also formed at 2960 cm^{-1} indicating a carbon hydrogen bond (CH). The mass spectrum of this compound indicates a series of fragments up to a mass of 107 and possibly greater.

The marine isolate of Desulfovibrio growing on the trypticase soy, ferrous ammonium sulfate, sea water agar also produces a gas with some similarities in the mass spectrum. As the traps that were used in collection of the compound had "greased" joints it is planned to collect the gas in a grease free system before attempting to determine the tentative structure of the compound based on the mass ratios.

(e.) Exposure of Mild Steel to Atmospheres of API Strain

To ascertain whether the volatile phosphorous compound would react with mild steel, a sterile 1010 steel coupon, submerged in a petri plate of sterile 2% yeast extract broth (pH7.0 $\pm$ 0.1) was placed in a Brewer jar under a hydrogen atmosphere with inoculated (API strain) plates of yeast extract plus phosphate agar (same medium used for isolation of the compound).

After two weeks inculation at 27°C, a black film was noted on top of the yeast extract broth. Upon further inculation (43 days total) the black material was removed, washed with distilled water and dried under vacuum at room temperature. The resulting black powder was non magnetic. After heating the material to 1,204°C in a vacuum oven for 15 minutes and allowing it to cool slowly to ambient temperature it was magnetic. X-ray diffraction revealed it to be FeS (hexagonal and orthorhombic forms). Upon treatment with dilute HCl (10%) some of the material went into solution accompanied by the odor of H_2S. X-ray diffraction of the resulting washed and dried material indicated the orthorhombic form of FeS was removed.

Lead sulfide was formed from a solution (10%) of lead sulfide exposed to the atmosphere of the organisms in another experiment.

As no phosphides were found in either case, it indicated no reaction of the volatile phosphorous compound, with either the steel or the lead salt under these closed conditions.

(f.) Reaction of Volatile Phosphorous Compound With Benzyl Viologen

Previously, it was reported (4) that bacteria-free filtrates of the API culture (Desulfovibrio) reduced the redox dyes, benzyl viologen (BV) (E_o' =-315mV) and methyl viologen (E_o' =-411mV) in a hydrogen atmosphere. As no reduction of the dye occurred in a inert nitrogen atmosphere, the reaction appeared similar to the enzymatic reaction catalyzed by a hydrogenase. Hydrogenase is an enzyme (protein in composition) capable of activating molocular hydrogen and catalysing the transference of hydrogen and/or electrons to a suitable acceptor, which may be another enzyme or a redox dye.

The action of the volatile phosphorous compound on iron suggested that it might be identical to the substance in the bacterial culture filtrate which reduced the two dyes in the presence of hydrogen.

In a preliminary experiment, an uninoculated plate of trypticase soy agar containing BV was placed in a Brewer jar under hydrogen with 9 inoculated plates (API strain) of 2% yeast extract agar (2%) containing K_2HPO_4 (1 gm) 200 ml agar. After a weeks inoculation period it was noted that the agar containing the BV became slightly darker than normal but no definite reduction of the BV occurred. After several more weeks incubation a few small areas of BV reduction began to form in the agar. No organisms (contaminants) appeared to be responsible for this reduction.

Upon removal of the plate, and addition of a drop of sodium hydroxide solution to the agar surface temporary reduction of the BV occurred. This suggested that the reduction of BV in this system was probably dependent on the pH of the medium.

A series of seven uninoculated tubes of 2% yeast extract broth (5 ml) containing BV (0.01%) and varying in pH from 7.2 to 9.6 (pH 7.2, 7.7, 8.3, 8.5, 9.1, 9.4, 9.6) were prepared using NaOH.

The tubes were placed in a vacuum desiccator under a H_2 atmosphere along with 9 inoculated (API strain) plates of 2% yeast extract agar plus K_2HPO_4 (0.5%). After 3 weeks incubation at 27°C, partial reduction of the BV was observed in the tubes. The degree of reduction was related to the pH. No reduction was noted in the broth at pH 7.2, slight reduction at pH 7.7 and increasing degrees of reduction in the remainder of the tubes corresponding to increase in the pH of the broth. The color of the broth in which reduction occurred was light blue rather than violet in color which is normally characteristic of reduced BV produced by filtrates (pH 8 ± 0.2) of yeast extract culture of Desulfovibrio containing steel. In a similar type of experiment, 9 plates of trypticase sea water agar plus Mohr's salt* (0.1 gm/200 ml) inoculated with the marine strain of Desulfovibrio were placed in a Brewer jar with two plates each containing BV (0.01%) in (10 ml) of (0.01m) tris (hydroxy-methyl aminomethane) buffer solution. The solution in one plate was neutralized to pH 7.0 with HCl. The other solution was not neutralized (pH 10.4).

After 1 week incubation the BV in the non neutralized tris solution was observed to be very slightly reduced. Upon further incubation the solution became decolorized again and in both solutions a white precipitate was observed. Evidently the tris buffer was precipitated by some reaction either reaction with H_2S or the volatile phosphorous compound. The pH of the solution originally at 10.4 was 6.0 and the buffer originally at 7.0 was 5.7.

*Ferrous ammonium sulfate.

It is hoped to investigate further the BV reduction by the volatile phosphorous compound to determine whether a phosphate acceptor may be involved in this reaction. It is possible that phosphorous may be split off from the compound simultaneously with its reduction by hydrogen and oxidation by BV.

3. Corrosion of Steel By the Marine Isolate of Desulfovibrio

One of the objectives of this contract was to study the effect of marine sulfate reducers on corrosion.

Polarization techniques for measuring the corrosion rate were employed, first because the corrosion rates were expected to be too small for accurate weight loss determination and secondly, a better understanding of the corrosion process would be possible.

(a.) Corrosion Cell

The corrosion cell (Figure 2) consisted of a 400 ml beaker (tall form) with a rubber stopper to support the electrodes and a Luggin capillary (Figure 3). The working or test electrode consisted of a cylinder of cold rolled 1020 steel 1/4" in diameter and 1/4" in length supported by a threaded rod. All of the surface except the end and edges of the cylinder was masked off by use of heat shrinkable plastic tubing. The exposed surface of the end of the cylinder was polished using progressively fine grades of emory paper (220, 320, 400, and 600) and, degreased in acetone. The electrode with the tubing was stored in 95% ethanol until ready for use. Just before use the residual alcohol was removed by flaming.

The platinum electrode (auxillary) consisted of a piece (6 X 7 mm) of platinum gauge (52 mesh to inch) attached to a platinum wire sealed in glass tubing. The platinum electrode was cleaned by flaming, acid washing (conc. HCl),

and rinsing in distilled water. The Luggin capillary tube consisted of a Pasteur capillary pipette bent so that the opening was a few mm from the face of the electrode.

In preparation of the cell, 250 ml of trypticase sea water medium (Appendix) minus the ferrous ammonium sulfate was sterilized in the beaker by autoclaving at 15 psi for 15 minutes. After removal from the autoclave, the medium was cooled rapidly by placing the beaker in cold tap water. The stopper holding the platinum electrode and the glass capillary tube were also sterilized by autoclaving. After removal from the autoclave, the capillary tube was filled with sterile, saturated KCl-agar (30 gm KCl, 3 gm agar, 100 ml dist. H_2O). After the KCl-agar had hardened, the working electrode was slid into an opening in the stopper and electrodes placed in the medium.

To prevent oxygen from dissolving in the medium, sterile autoclaved (1 hr, 15 psi) melted vaspar (equal parts of vaseline and paraffin) was poured on the surface of the medium through a hole in the stopper to a depth of about 1 inch. The hole was then stopped and the beaker placed in an incubator (27°C). In addition to providing a relatively constant temperature, the cell was shielded from light (1) and stray currents.

A salt bridge (tubing containing KCl agar) was connected from the end of the capillary tube to a beaker containing saturated KCl solution and a calomel electrode. Nitrogen was slowly bubbled through the solution in the beaker for a few weeks to prevent diffusion of oxygen through the bridge. The outlet was than sealed.

Polarization and redox measurements were made for about one week prior to introduction of the organisms, to insure sterility and to obtain the background rate of corrosion.

After an interval of one week, when it was determined that the medium was sterile (absence of turbidity and gas), a small hole was made through the vaspar by a heated rod and 0.5 ml of inoculum introduced by a hypodermic syringe. The inoculum consisted of the 5 day old growth of the marine strain of Desulfovibrio, removed with a bacteriological loop, from the surface of two plates [agar medium used for cultivation of the marine strain, (Appendix)] and suspended in 1 ml of sterile aged sea water.

(b.) Redox Potential Measurements

Redox potential measurements were calculated by adding +0.241 volts to the observed reading of the platinum electrode (ref: sat. calomel electrode). No corrections for pH were made as the pH of the medium was initially adjusted to 7.0 ± .01 and the pH of the culture after removal of the electrodes was 6.9.

(c.) Measurement of Corrosion Rate

Corrosion rates were determined by two polarization methods, the "polarization resistance" or "polarization admittance" method and the "polarization break" method.

The "polarization admittance" technique is based on the following relationship derived by Stern and Geary (5):

$$\frac{\Delta E}{\Delta I} = \frac{1}{2.3\ I_{corr}} \left(\frac{B_a\ B_c}{B_a + B_c}\right)$$

In this equation ΔE is the overvoltage of the corroding electrode produced by a polarizing current ΔI. $\Delta E/\Delta I$ is the slope of the polarization curve

(termed "polarization resistance"), B_a and B_c are the slopes of the anodic and cathodic polarization curves in the Tafel region and $I_{corr.}$ is the corrosion current.

The ratio, $\Delta I/\Delta E$, rather than the inverse, as in the above equation, was used since this ratio is directly proportional to the corrosion rate ($I_{corr.}$) as indicated below:

$$\frac{\Delta I}{\Delta E} = 2.3\ I_{corr}\ \left(\frac{B_a+B_c}{B_a\ B_c}\right)$$

The ratio $\Delta I/\Delta E$ has been called "polarization admittance" by some investigators.

The constant B_a and B_c were both assumed to be equal to 0.1 in this investigation. This results in the following equation:

$$I_{corr.}\ (mA) = \frac{1}{.0023}\ \left(\frac{\Delta I}{\Delta E}\right)\ \left(\frac{B_a\ B_c}{B_a+B_c}\right)$$

or

$$I_{corr.}\ (mA) = \frac{21.7\ \Delta I(mA)}{\Delta E(mV)}$$

The "polarization break" method was referred to in the last report (1). It is based on Pearson's derivation (6) of the equation:

$$I_{corr.} = \frac{I_a\ I_c}{I_a+I_c}$$

when $I_{corr.}$ is the corrosion rate and I_a and I_c represent the current values at breaks or changes in the slopes of the anodic and cathodic polarization curves respectively.

The electrical circuit employed was essentially similar to that shown on page 39 of the previous report (1) except that a variable resistance of 6 megohms was inserted in series in the current supply circuit to the electrodes. Potential measurements were made with an electronic potentiometer. Current measurements were made with a multiple range microammeter.

Each time the measurements were made, the open circuit potentials of the platinum electrode (redox potential) and the steel electrode were first obtained. An increment of current sufficient to polarize the electrode to an overpotential of about 10 mV was then measured to calculate the corrosion rate by the Stern equation. The current was cut off and after an interval of about two hours (usually sufficient for depolarization) a cathodic polarization curve was made using the galvanostatic technique with 2 minutes intervals between increasing equal increments of current. Previous calculation of the corrosion current was found to be extremely helpful in deciding what equal current increments to use.

After a sufficient time interval, usually several hours, the anodic polarization curve was made. The steel electrode was never polarized to greater than 100 mV in either direction, usually 60 or 70 mV.

(d.) Results

The results of these determinations are presented in Table 1. As indicated in the table, the open circuit potential of the steel moved over 200 mV to a more noble value and then changed to a more "active" value (more negative).

The redox potential dropped to over 400 mV simultaneously with active growth of the culture and then began a slow rise. The corrosion rate was found to increase slightly on the 1st day after inoculation, probably due to the introduction of some air along with the inoculum, and then decreased steadily until the 25th day when a slight increase was noted. Upon further passage of time the corrosion rate increased until it was about 6 times that of the corrosion rate before inoculation as determined by the polarization

break method. Coincident with the decrease and increase in corrosion rate was an increase and decrease $\frac{\Delta V}{\Delta I}$ (polarization resistance of the slopes following the break). From the 26th day on until the 42nd day the open circuit potentials were very unsteady for some reason and no polarization data were obtained.

The polarization curves up to and 1 day after inoculation of the organisms were similar to those in Figure 4a. The break in the cathodic curve occurred before the break in the anodic curve. In addition the slope in the anodic curve (noble direction) after the break was always found to be less than the slope of the curve before the break. These types of curves appear to be typical of those usually obtained from iron buried in soils. On the second and third day after inoculation when the organisms were growing rapidly as evidenced by development of turbidity and a lowering in the redox potential, the polarization curves were typical of those in Fig. 4b. The break in the anodic curve occurred before the break in the cathodic curve and the slope in the anodic curve was greater after the break than before. From the 4th to the 14th day curves typical of those shown in Fig. 4c were obtained. The break in the cathodic curve again occurred before the break in the anodic curve as in Fig. 4a, but the slope of the anodic curve after the break was still greater than the slope before the break as in Fig. 4b. From the 16th day to and including the 23rd day after inoculation the polarization curves were typical of those in Fig. 4b. On the 25th day, the curves were again similar to those in Fig. 4a. Curves typical of Fig. 4c were obtained for the remainder of the days the curves were obtained.

(e.) Discussion

These observations, namely the decrease in corrosion rate, the change in potential of the steel to a more noble direction and the increase in polarization resistance appear to be indicative of the formation of a visible protective. Reversal of these changes indicated that the film was breaking down.

The appearance of the steel surface (Fig. 5) tended to confirm these indications. As shown in Fig. 5, a black film covered most of the surface except near the edge of almost one half the specimen. Breakdown of the film in these areas probably contributed to the increase in rate of corrosion and associated rise to a more active potential. Examination of Fig. 3 shows the loose black film becoming detached as evidence by the irregular outline at the bottom of the electrode.

Further examination of the film by the electron-probe technique revealed considerable iron, a fair amount of sulfur and very little phosphorous. The film mainly consisted of iron sulfide (FeS). It is hoped upon further investigation to determine more precisely the nature of the film.

Similar observations were made by Booth (6). In corrosion (weight loss) experiments in batch cultures with periodic renewal of the nutrient medium he observed initially low corrosion rates consistant with the formation of a thin hard coating which afforded considerable protection. "After a period of some weeks the coating became detached in an irregular manner, the corrosion rate increased rapidly and the metal acquired the general appearance typical of bacterial corrosion in practice, i.e. loose black corrosion product overlying bright metal". The extent of the initial hard coating and the rate at which it became detached were reported by Booth to depend on the strain of organism used.

It was possible that different strains of organisms may produce different ratios of the phosphorous compound and hydrogen sulfide. Both compounds would be expected to produce films which may differ considerably in resistance to attachment.

It is hoped to investigate this further. The Seitz filtrate (bacteria-free) of the culture apparently had very little of the phosphorous compound judging from the fact that reduction of the benzyl viologen by the filtrate took many hours. The API culture filtrate which produces Fe_2P readily reduces benzyl viologen in a matter of minutes.

Some cathodic and anodic depolarization did occur early, as was evident by a decrease in the $\frac{\Delta E}{\Delta I}$ ratio on the 3rd and 4th day, and a slight increase in the corrosion rate was noted ("P.A" method). The greatest increase in the corrosion rate accompanied by anodic and cathodic depolarization however occurred as the film was detaching. A condition would then exist in which there are iron and iron sulfide electrodes. It has been suggested by Stumper (8) that such corrosion cells may be a possible cause of corrosion. Quite recently Booth et al (9) have found considerable evidence that ferrous sulfide causes considerable depolarization of the cathode. They stated that this mechanism may make a major contribution to the overall anodic corrosion process. In an earlier report Booth et al (10) using semi-continuous cultures had observed very high rates (>50 $mg/dm^2/day$) in minimal iron medium which were not experienced in iron rich medium. They stated: "It seems probable that, in minimal iron medium, after the temporary protective film that forms has been removed, the corrosion is stimulated both by depolarizing action at the cathode and by sulfide action at the anode. In the iron rich medium experiments, the sulfide is continuously removed from solution and only the cathodic depolarizing stimulus remains."

The corrosion rates as measured by the "polarization admittance" method were several times higher in many cases than those as determined by the polarization break method. The values as determined by polarization admittance are maximum values and would have decreased with an increase in time. A further increase in potential might have occurred with a long period of polarization. Several hours polarization time was allowed as the corrosion rates became very low. In addition the beta values were assumed to be equal to 0.1. To determine these values (Tafel slopes) it is recommended that polarization of the electrode be made over several orders of current density (11). It was feared that this might have an adverse effect on the bacteria or the electrode process or both.

SUMMARY

1. An agar plating medium which allows good surface growth of marine sulfate reducers (Desulfovibrio) has been devised. It consists of trypticase, phytone and aged sea water. Ferrous ammonium sulfate may be added as an indicator salt for selective isolation. Hydrogen sulfide appears to be produced in considerable amounts by the organisms when growing on plates of this medium.

2. A marine isolate of Desulfovibrio has been obtained in pure culture through use of this medium.

3. Growth of four non-marine forms of Desulfovibrio on the surface of an agar medium used for maintenance is greatly stimulated by the substitution of aged sea water for distilled water.

4. Growth of the marine strain in a liquid medium (same ingredients as the plating medium) in the presence of ferrous ions results in formation of a black material which is a mixture of iron sulfide and iron phosphide (Fe_3P; schreibersite).

5. Identification of the yellow orange colored oxidation product by X-ray diffraction has not been successful.

6. Preliminary studies on the corrosion of steel by the marine isolate of Desulfovibrio using polarization techniques indicates a decrease in corrosion rate, followed by a increase. These changes appears to be associated with the formation and dissolution of a iron sulfide film. The major portion of anaerobic corrosion may be due to disruption of this film producing a iron sulfide couple. Iron sulfide has been shown by others to greatly increase the anaerobic corrosion rate of steel.

7. A non-marine strain of Desulfovibrio has been demonstrated to produce a volatile organo-phosphorous compound which appears to be responsible for the production of iron phosphide (Fe_2P) and the reduction of benzyl viologen, a redox dye. It also appears to be produced by the marine strain.

Acknowledgment

Work on the identification of the organo-phosphorous compound has been done in co-operation with Dr. F. Brinckman. Inorganic Chemistry Section, National Bureau of Standards. X-ray diffraction studies were done by Mr. C. Bechtold, Metallurgy Division, National Bureau of Standards. Mossbauer studies by Dr. L. Bennett and electron probe studies expedited by Mr. H. Yakowitz, both in the Metallurgy Division, National Bureau of Standards are also most gratefully appreciated.

REFERENCES

1. Tech. Sum. Rept. No. 1 National Bureau of Standards Rep't. No. 9846.
2. W. P. Iverson, J. Appl. Microbiol 14 (4) 529, (1965).
3. J. R. Postgate, Prod. Month, 22 (9), 12 (1958).
4. W. P. Iverson, Nature 217, 1265 (1968).
5. M. Stern and A. Geary, J. Electrochem. Soc. 104, 56 (1957).
6. J. M. Pearson, Trans. Electrochem. Soc., 81, 485 (1942).
7. G. H. Booth, J. Appl. Bacterial 27, 174 (1964).
8. R. Stumper, Compt. Rend. 176, 1316 (1923).
9. G. H. Booth, L. Elford, and D. S. Wakerly, Br. Corros. J. 3, 242 (1948).
10. G. H. Booth, P. M. Shinn and D. S. Wakerly, Congr. int. Cor. Marine et des Salissures p. 363, (Paris: CREO) (1964).
11. N. D. Greene, Experimental Electrode Kinetics, Rensselaer Polytechnic Inst. Troy, New York (1965).

APPENDIX

Trypticase Soy Agar Plus Salts Medium*

+Trypticase Soy Agar (BBL)	40.0 gm
Sodium lactate (60% sol.)	4.0 ml
Magnesium sulfate	2.0 gm
Ferrous ammonium sulfate	0.5 "
Agar	5.0 "
Distilled water	1000.0 ml

Ferrous ammonium sulfate solution (10 ml dist. H_2O) sterilized by Seitz filtration. pH adjusted to 7.0 ± 0.1.

+Trypticase Soy Agar (BBL)*

Trypticase	15.0 gm	(pancreatic digest of casein)
Phytone	5.0 "	(Papaic digest of soya meal)
Sodium Chloride	5.0 "	
Agar	15.0 "	
Distilled water	1000.0 ml	

*Baltimore Biological Laboratories

American Petroleum Institute Medium*

Sodium lactate (60%)	4.0	ml
Yeast extract	1.0	gm
Ascorbic Acid	0.1	"
$MgSO_4.7H_2O$	0.2	"
K_2HPO_4 (Anhyd)	0.01	gm
$Fe(SO_4)_2$ $(NH)_2.6H_2O$	0.1	"
NaCl	10.0	"
Distilled water	1000.0	ml

pH adjusted to 7.2 - 7.5 with NaOH

Sea-Salts (ASTM)•

	%
NaCl	58.490
$MgCl_2.6H_2O$	26.460
Na_2SO_4	9.750
$CaCl_2$	2.765
KCl	1.645
$NaHCO_3$	0.477
KBr	0.238
K_3BO_3	0.071
$SrCl_2.6H_2O$	0.095
NaF	0.007

41.953 gm per liter H_2O

*R.C. Allred, Prod. Month. 22, 32 (1958)

•Lake Products Co., St. Louis, Mo.

Agar Medium for Growth of Marine Sulfate Reducers

Trypticase Soy Agar (BBL)	40.0 gm
Agar	5.0 "
Ferrous ammonium sulfate	0.5 "
Aged sea water	995.0 ml

pH adjusted to 7.0 ± 0.1. Ferrous ammonium sulfate solution (0.5 gm/5 ml dist. H_2O) sterilized by Seitz filtration and added to autoclaved medium before pouring plates.

Trypticase Soy Broth (BBL) plus Agar

Trypticase	17.0 gm
Phytone	3.0 "
Sodium chloride	5.0 "
Dipotassium phosphate	2.5 "
Dextrose	2.5 "
Distilled water	1000.0 ml

Agar to a conc. of 2%.

pH 7.3 ±

Trypticase Sea Water Medium

Trypticase	15.0 gm
Phytone	5.0 "
Sodium chloride	5.0 "
Ferrous ammonium sulfate	2.5 "
Sea water	980.0 ml

pH adjusted to 7.0 ± 0.1 with NaOH. Ferrous ammonium sulfate solution (2.5 gm/20 ml dist. water), Seitz filtered, was added immediately after sterilization.

Table 1

Instantaneous Corrosion Rates of 1020 Steel In a Trypticase - Sea Water Culture of A Marine Isolate of Desulfovibrio

Cumulative Time (Days)	Open Circuit Pot. of Steel[a] (V)	Redox Pot. (V)	Polarization Current At Break Cathodic Ic (μA)	Polarization Current At Break Anodic Ia (μA)	Corrosion Rate "PB" Method[b] Current Density (μA/cm²)	Corrosion Rate "PB" Method[b] Wt. Loss (mdd)[c]	Cathodic[d] ΔE/ΔI (X10³ohms)	Anodic[d] ΔE/ΔI (X10³ohms)	Corrosion Rate "PA" Method[e] Current Density (μA/cm²)	Corrosion Rate "PA" Method[e] Wt. Loss (mdd)[c]
1[f]	-.717	+.261	0.42	0.46	0.70	1.75	100	20	1.12	2.80
2	-.719	+.258	0.32	0.43	0.58	1.45	66	20	1.47	3.68
3	-.718	+.222	0.15	0.50	0.38	0.95	70	20	0.96	2.40
4	-.711	+.241	0.30	0.30	0.48	1.20	68	20	1.38	3.45
7	-.699	+.252	0.25	0.30	0.42	1.05	35	16	2.69	6.72
1[g]	-.695	+.266	1.20	1.80	2.30	5.86	63	8	3.07	7.68
2	-.702	-.189	0.25	0.22	0.38	0.95	100	62	0.70	1.75
3	-.618	-.202	0.20	0.12	0.26	0.65	54	65	1.15	2.88
4	-.604	-.208	0.18	0.25	0.33	0.82	45	62	1.02	2.55
7	-.564	"	0.16	0.19	0.28	0.70	166	175	0.34	.85
8	-.604	"	0.14	0.15	0.23	0.57	125	162	0.70	1.75
9	-.581	"	0.08	0.16	0.17	0.42	160	275	0.31	.77
11	-.565	"	0.08	0.10	0.14	0.35	225	340	0.39	.98
14	-.550	-.209	0.07	0.10	0.13	0.32	225	314	0.26	.65
16	-.540	-.207	0.07	0.06	0.10	0.25	340	333	0.07	.17
18	-.530	-.220	0.06	0.04	0.08	0.20	514	333	0.06	.15
21	-.491	-.203	0.03	0.02	0.04	0.10	322	307	----	----
23	-.474	-.201	0.12	0.07	0.13	0.32	433	293	0.05	.12
25	-.451	-.206	0.16	0.13	0.22	0.55	340	81	0.06	.15
42	-.548	-.192	0.83	1.50	1.73	4.32	35	35	----	----
43	-.582	-.189	1.36	1.96	2.56	6.40	27	22	1.53	3.80
44	-.574	"	----	----	----	----	---	---	1.73	4.30
49	-.605	-.187	----	----	----	----	---	---	1.38	3.40
50	"	-.189	----	----	----	----	---	---	2.56	6.40
51	-.604	-.189	1.70	2.42	3.2	8.00	16	23	2.88	7.20

[a] Ref: Sat calomel half cell

[b] "Polarization break" method

$$I_{corr.} = \frac{I_c I_a}{I_c + I_a}$$

Current density ($\mu A/cm^2$) = $I_{corr}/.31\ cm^2 \times 3.2$

[c] mdd = milligrams per square decimeter per day

[d] $\frac{\Delta V}{\Delta I}$ = slope of curve past "break" point

[e] "polarization admittance" method

$$I_{corr}\ (mA) = \frac{21.7\ \Delta I(mA)}{\Delta E(mv)}$$

[f] Days after cell prepared (medium sealed with vaspar)

[g] Days after introduction of organisms in cell

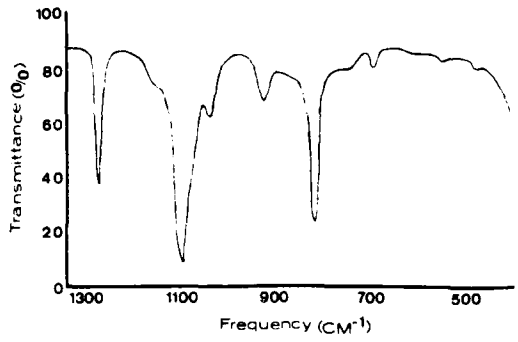


Fig. 1. IR spectra of volatile phosphorous compound.

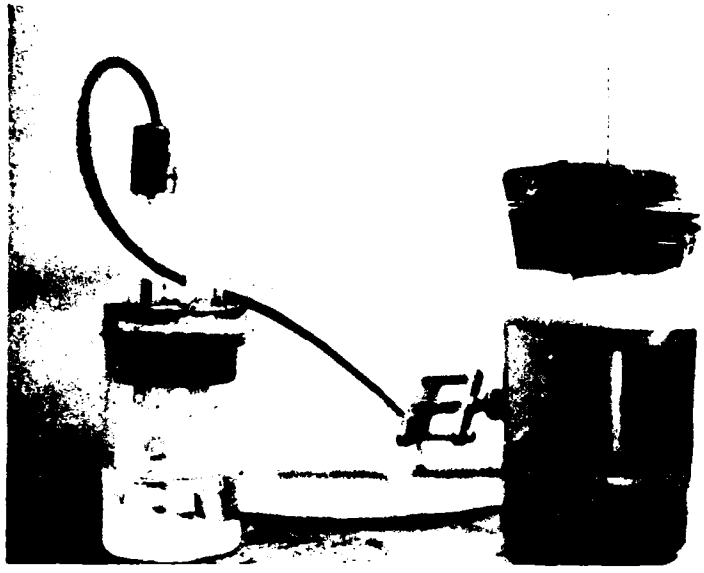


Fig. 2. Polarization cell before removal of electrodes.

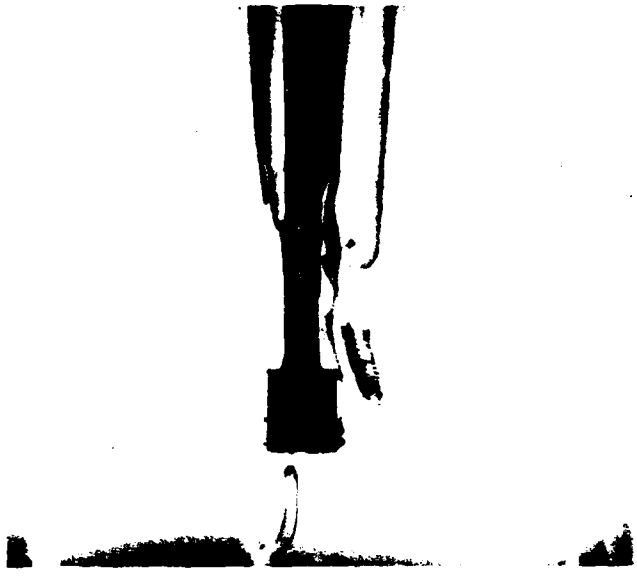
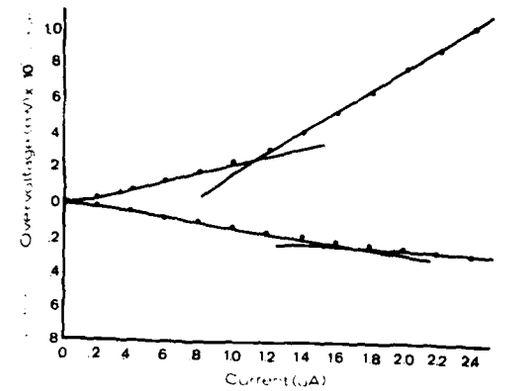
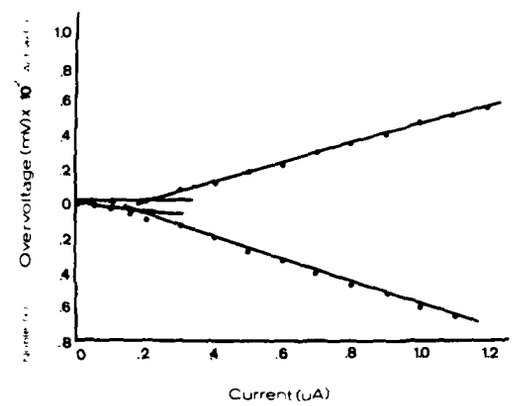


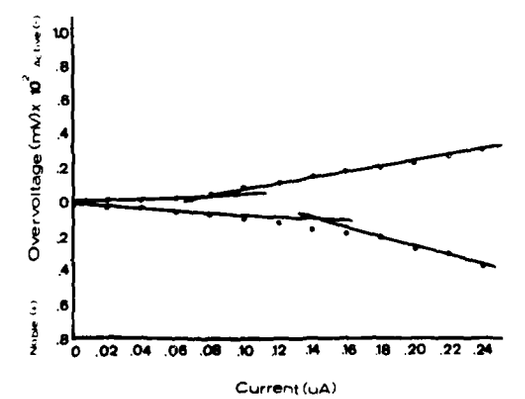
Fig. 3. Close up view showing electrodes just before removal.



a



b



c

Fig. 4. Polarization curves of 1020 steel in inoculated trypticase sea water medium: a) 1 day after inoculation, b) 3 days after inoculation and, c) 9 days after inoculation.

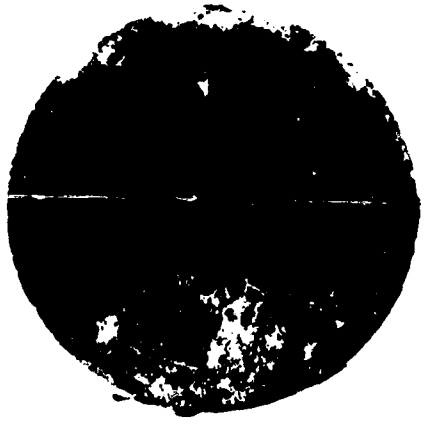


Fig. 5. Face of 1020 steel electrode after removal from culture showing dark film and areas at the top edge where the film has broken away from surface. White area at bottom due to drop of KCl-agar which fell on specimen from Luggin capillary during drying of film (10X magnification).

UNCLASSIFIED
Security Classification

DOCUMENT CONTROL DATA - R & D

Security classification of title, body of abstract and indexing annotation must be entered when the overall report is classified.

1. ORIGINATING ACTIVITY (Corporate author) National Bureau of Standards Washington, D. C. 20234	2a. REPORT SECURITY CLASSIFICATION Unclassified 2b. GROUP

3. REPORT TITLE
Microbial Corrosion

4. DESCRIPTIVE NOTES (Type of report and inclusive dates)
Technical Summary Report No. 2

5. AUTHOR(S) (First name, middle initial, last name)
Warren P. Iverson and Joan Calvert

6. REPORT DATE	7a. TOTAL NO OF PAGES	7b. NO OF REFS
1 May 1969	32	11

8a. CONTRACT OR GRANT NO	9a. ORIGINATOR'S REPORT NUMBER(S)
NAONR 14-67, NR 137-793 b. PROJECT NO 3120442 c d	3120442 - Technical Summary Report No. 2 9b. OTHER REPORT NO(S) (Any other numbers that may be assigned this report)

10. DISTRIBUTION STATEMENT
Distribution of this document is unlimited

11. SUPPLEMENTARY NOTES	12. SPONSORING MILITARY ACTIVITY
	Office of Naval Research

13. ABSTRACT

An agar medium which will support good surface growth of marine sulfate reducers (Desulfovibrio) has been devised. Using this medium, a marine isolate of Desulfovibrio has been obtained in pure culture and used for anaerobic corrosion studies. Using polarization techniques for corrosion rate determinations, it was found that the rate of corrosion of mild steel (1020), in a culture of the same medium minus added ferrous ions, decreased to about 0.1 mdd and then increased to 7-8 mdd before termination. These changes were correlated with the formation and removal of an iron sulfide film.

A non-marine strain of Desulfovibrio was found to produce a volatile organo-phosphorous compound which reacts with mild steel to form iron phosphide (Fe_2P). It also has the ability to reduce the redox dye, benzyl viologen. The marine isolate also appears to produce this compound.

Growth of the marine strain in a sea water medium containing ferrous ions to which trypticase and phytone were added resulted in formation of a black material, which upon heating to 1,204°C in a vacuum oven, was found to consist of a mixture of FeS and Fe_3P (schreibersite).

DD FORM 1 NOV 1473 (PAGE 1)

S/N 0101-807-6801

Security Classification

Security Classification

14 KEY WORDS	LINK A		LINK B		LINK C	
	ROLE	WT	ROLE	WT	ROLE	WT
Microbiological corrosion						
Marine corrosion						
Cathodic depolarization						
Sulfate reducers						
Desulfovibrio sp.						
Iron phosphide						
Iron sulfide						
Hydrogen sulfide						
Polarization resistance						
Polarization admittance						
Redox-potential						
Schreibersite						

DD FORM 1 NOV 68 1473 (BACK)
(PAGE 2)

Security Classification